DETBA Value and Hexanal Production with the Combination of Unsaturated Fatty Acids and Extracts Prepared from Soybean Seeds Lacking Two or Three Lipoxygenase Isozymes

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Hydroperoxide and *n*-hexanal production by lipoxygenase action is an important problem in the effective use of soybean seeds for food ingredients. We examined here the additional effect of unsaturated fatty acids on the 1,3-diethyl-2-thiobarbituric acid (DETBA) value (index for hydroperoxide production) and *n*-hexanal production with two normal soybean cultivars; three mutants partially lacking lipoxygenase and containing only L-1, L-2, or L-3; and one mutant completely lacking lipoxygenase (L_0). Among the soybeans tested, the L_0 soybean had the lowest levels of both DETBA value and *n*-hexanal production. The low DETBA values were maintained over a wide range of pH (4−10) and temperature (4−70 °C) and were not influenced by the addition of unsaturated fatty acids. These results suggest that the L_0 soybean could be an excellent soybean source for making soybean products with a combination of soybean and other food ingredients.

Keywords: *Soybean; lipoxygenase isozymes; hexanal; DETBA value; lipid peroxidation; unsaturated fatty acid; food ingredient; Glycine max*

INTRODUCTION

One of the major obstacles to the use of soybeans as food ingredients is the development of undesirable grassy-beany flavor and bitter taste. Several authors have shown evidence that volatile and nonvolatile constituents responsible for the off-flavor are generated by hydroperoxidation of certain unsaturated fatty acids and various polyunsaturated lipids containing *cis*,*cis*-1,4-pentadiene moieties through the action of lipoxygenase and subsequent cleavage of the products by hydroperoxide lyase (Rackis et al., 1979; Matoba et al., 1985a,b; Hildebrand, 1989). In fact, normal commercial soybean cultivars with high levels of lipoxygenase and linoleic acid produce unacceptable amounts of *n*-hexanal. Therefore, the hydroperoxide production by lipoxygenases is an important problem in the effective use of soybean seeds for food ingredients (Kaneda et al., 1955; Ames, 1983; Haumann, 1993).

Normal soybean seeds contain three types of lipoxygenase isozymes, called lipoxygenase 1 (L-1), lipoxygenase 2 (L-2), and lipoxygenase 3 (L-3) (Axelrod et al., 1981). The action of these isozymes is so strong and rapid that oxidation of unsaturated acids proceeds instantaneously when soybean seeds are crushed into meal. Thus, genetic elimination of lipoxygenase from soybean seeds has attracted attention as a useful approach to prevent the enzymatic hydroperoxide production. So far, several types of soybeans lacking lipoxygenase have been produced; for examples, L-1 null (Hildebrand and Hymowitz, 1981), L-2 null (Kitamura et al., 1985; Davies and Nielsen, 1986), L-3 null (Kitamura et al., 1983), double L-1 and L-2 null (Hajika et al., 1992), double L-1 and L-3 null and double L-2 and L-3 null (Kitamura et al., 1985), and triple L-1, L-2, and L-3 null (Hajika et al., 1991). Using some of these soybeans lacking lipoxygenase, we previously demonstrated that production of hydroperoxide and *n*-hexanal was the lowest in "Kyushu 111" (triple-null) and relatively lower in "Yumeyutaka" (double L-2 and L-3 null) and "Kyushu 119" (double L-1 and L-2 null). We also showed some evidence that "Kyushu 111" became an excellent food ingredient for making soybean products consisting of soybean seeds alone, such as soymilk and tofu (Nishiba et al., 1995). Thus, it would be interesting to determine whether and to what extent the production of hydroperoxide and *n*-hexanal in such lipoxygenase-line soybeans was elevated by the external addition of unsaturated fatty acids because the finding of soybean seeds with low productivity of both compounds in the presence of unsaturated fatty acids means the birth of new types of soybean products, combining soybean seeds and other food ingredients. This combination would result in greatly expanded utilization of soybean seeds.

The triple-null soybean seed "Kyushu 111" is a strong candidate with superior traits. Thus, we examined here the 1,3-diethyl-2-thiobarbituric acid (DETBA) value (index for hydroperoxide production; Suda et al., 1994; Furuta et al., 1995) and *n*-hexanal production with the combination of unsaturated fatty acid and soybean extracts from triple-null soybeans and compared the results with results from other soybean extracts.

MATERIALS AND METHODS

Soybeans. Two normal soybean [*Glycine max* (L.) Merr.] types and four lipoxygenase-lacking mutants were used in this study. Suzuyutaka and Fukuyutaka were normal soybean cultivars containing L-1, L-2, and L-3 isozymes (abbreviated as $L_{123(S)}$ and $L_{123(F)}$ based on the remaining isozyme phenotype). Yumeyutaka (L_1), Kanto 102 (L_2), and Kyushu 119 (L_3) were mutants containing only one isozyme; that is, L-1, L-2, and L-3, respectively. Kyushu 111 (L_0) was a mutant lacking all three types of lipoxygenase isozymes. The presence or absence of lipoxygenase isozymes in their soybean seeds was confirmed by SDS-PAGE and a spectrophotometric method (Nishiba et al., 1995). The mutants lacking lipoxygenase were derivatives of Suzuyutaka. These soybeans were harvested at the Kyushu National Agricultural Experiment Station in 1992-1993.

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0021-8561/96/1444-0236$12.00/0

Reagents. DETBA was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Linoleic acid, linolenic acid, and arachidonic acid were obtained from Sigma Chemical Company (St. Louis, MO). Butyl hydroxy toluene (BHT) and 2,4-dinitrophenylhydrazone were obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan). The other chemicals were analytical grade reagents.

Preparation of Soybean Extract. Soybean seeds were soaked in 98 volumes of distilled water at 4 °C overnight, homogenized with a Polytron homogenizer (Kinematica, Switzerland) with cooling in an ice bath, and allowed to stand for 30 min at 4 °C. The supernatant obtained by centrifugation (10000*g*, 10 min, 4 °C) was used as the soybean extract sample within 1 h.

Preparation of Unsaturated Fatty Acids. Sodium linoleate (10 mM) was prepared from linoleic acid as described previously (Suda et al., 1995). Sodium linolenate and sodium arachidonate were prepared in the same manner with linoleic acid and arachidonic acid instead of linoleic acid.

Measurement of DETBA Value. Linoleate oxidation by soybean extract was performed at 25 °C in a test tube with a screw cap. To the test tube, 50 μL of 200 mM sodium phosphate buffer (pH 7.0), 10 μL of 10 mM sodium linoleate, 25 μL of soybean extract, and 15 μL of distilled water were added, and the tube was capped. The reaction was initiated by adding the soybean extract. After an appropriate incubation time (usually 10 min), 200 μL of 20 mM BHT, 200 μL of 8% SDS, and 300 μL of distilled water were added to stop the enzyme reaction, and the product was subjected to DETBA assay as described previously (Suda et al., 1994). The DETBA value was expressed as malondialdehyde per milligram of protein of soybean extract.

An experiment to examine the effect of pH on linoleate oxidation was carried out with acetate (pH 4−6), phosphate (pH 6−8), and borate (pH 8−10) buffers. The experiment to examine the effect of temperature on linoleate oxidation was done by incubating the reaction mixture at 4, 10, 20, 30, 40, 50, 60, and 70 °C. In some experiments, sodium linoleate was replaced by sodium linolenate or sodium arachidonate.

Determination of *n*-Hexanal. To a test tube with a screw cap, 500 μL of 200 mM sodium phosphate buffer (pH 7.0), 100 μL of 10 mM sodium linoleate, 250 μL of soybean extract, and 150 μL of distilled water were added and incubated for 10 min at 25 °C. *n*-Hexanal accumulated in the reaction mixture was determined as the 2,4-dinitrophenylhydrazone derivative by high-performance liquid chromatography (HPLC) as described previously (Nishiba et al., 1995).

Protein Contents. Protein content in the soybean extract was determined according to the method of Lowry et al. (1951), with bovine serum albumin as the standard.

RESULTS

Time-Dependent Increase in DETBA Value with the Combination of Linoleic Acid and Soybean Extract. The serial changes in DETBA value with the combination of linoleic acid and soybean extract are shown in Figure 1. The DETBA values at 0 min almost corresponded to the DETBA values of the soybean extract alone. External addition of linoleic acid to the soybean extracts induced the time-dependent increase in DETBA value with soybean extracts from $L_{123(S)}$, $L_{123(F)}$, L_1, L_2 or L_3, whereas the increase was not observed in the DETBA value from L_0. DETBA values with L_1, L_2, or L_3 were half the level of the DETBA value with $L_{123(S)}$ or $L_{123(F)}$. Because a considerable lipoxygenase mutation-derived difference in the DETBA value was found in samples incubated for 5−60 min with the combination of linoleic acid and soybean extract, subsequent experiments had a 10-min incubation period.

Effects of pH and Temperature on DETBA Value with the Combination of Linoleic Acid and Soybean Extract. A study of the pH profiles of linoleate oxidation by soybean extracts showed that a high

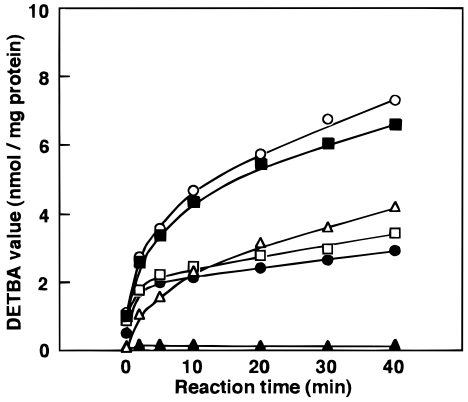

Figure 1. Time-dependent increase in DETBA value with the combination of linoleic acid and soybean extract. The reaction mixture in a total volume of 200 μL contained 100 mM phosphate buffer (pH 7.0), 1 mM sodium linoleate, and soybean extract. Incubation time was varied as indicated at 25 °C, and the obtained sample was subjected to DETBA assay. Results are expressed as means of three experiments: (■) Suzuyutaka ($L_{123(S)}$); (○) Fukuyutaka ($L_{123(F)}$); (△) Yumeyutaka (L_1); (□) Kanto 102 (L_2); (●) Kyushu 119 (L_3); (▲) Kyushu 111 (L_0).

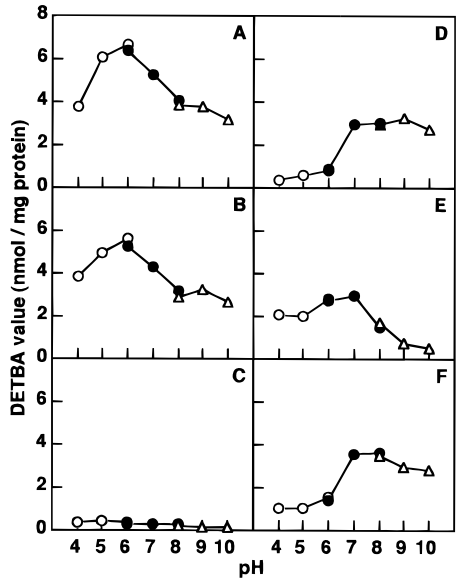

Figure 2. Effects of pH on the DETBA value with the combination of linoleic acid and soybean extract. The reaction mixture was as described in Figure 1, except that a 10-min incubation time was employed and the pH was varied as indicated with acetate (pH 4−6), phosphate (pH 6−8), and borate (pH 8−10) buffers. Results are expressed as means of three experiments: (A) Suzuyutaka ($L_{123(S)}$); (B) Fukuyutaka ($L_{123(F)}$); (C) Kyushu 111 (L_0); (D) Yumeyutaka (L_1); (E) Kanto 102 (L_2); (F) Kyushu 119 (L_3).

DETBA value was obtained at pH 6 in $L_{123(S)}$ and $L_{123(F)}$, at pH 7−9 in L_1, at pH 6−7 in L_2, and at pH 7−8 in L_3 (Figure 2). Their pH values were similar to the pH showing maximum activity of lipoxygenase isozyme, which was at pH 9 for L-1 and at pH 6−7 for L-2 (Axelrod et al., 1981). On the contrary, the DETBA value in L_0 was maintained at low levels at all pH ranges between 4 and 10.

A study of the temperature profiles of linoleate oxidation by soybean extract showed that the DETBA value in soybean extracts, except for L_2 and L_0, gradually increased along with temperature up to 50 °C and then decreased (Figure 3). L_2 had a high DETBA value even at 4 °C, and a maximum value at 30 °C instead of 50 °C. This result is probably due to the heat susceptibility of the L-2 isozyme. Temperature profiles of

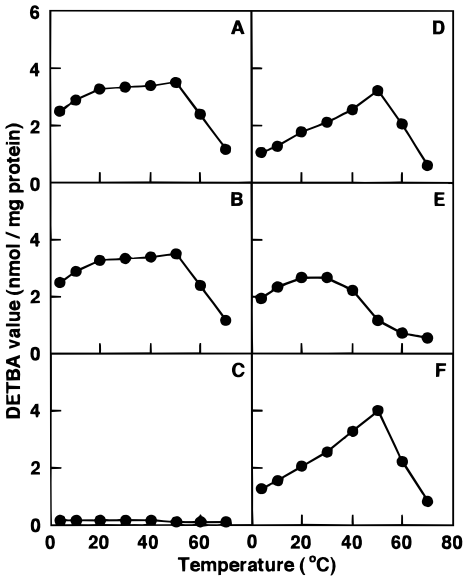

Figure 3. Effects of temperature on the DETBA value with the combination of linoleic acid and soybean extract. The reaction mixture was as described in Figure 1, except that a 10-min incubation time was employed and the temperature was varied as indicated. Results are expressed as means of three experiments: (A) Suzuyutaka ($L_{123(S)}$); (B) Fukuyutaka ($L_{123(F)}$); (C) Kyushu 111 (L_0); (D) Yumeyutaka (L_1); (E) Kanto 102 (L_2); (F) Kyushu 119 (L_3).

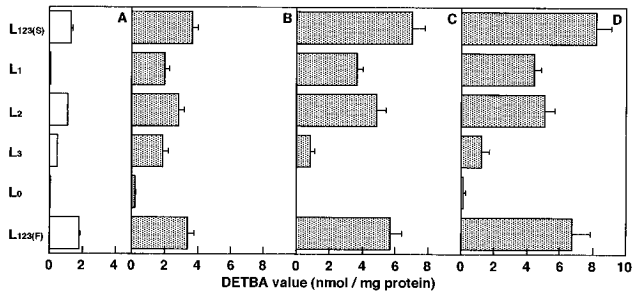

Figure 4. DETBA value with the combination of unsaturated fatty acids and soybean extract. The reaction mixture was as described in Figure 1, except that a 10-min incubation time was employed and linoleic, linolenic, or arachidonic acid was used as the substrate. Results are expressed as means of three experiments: (A) none; (B) linoleic acid; (C) linolenic acid; (D) arachidonic acid.

$L_{123(S)}$ and $L_{123(F)}$ contained in all three types of lipoxygenase isozymes seemed to be a mixed form of the temperature characteristics of L_1, L_2, and L_3; that is, the profiles reflected the characteristics of L_2 at low temperature and the characteristics of L_1 and L_3 at high temperature. On the contrary, the DETBA value in L_0 was maintained at low levels at all temperature ranges between 4 and 70 °C.

Thus, it was suggested that the pH and temperature profiles of each soybean may depend on the pH and temperature characteristics of the isozyme contained in it.

DETBA Value with the Combination of Unsaturated Fatty Acids and Soybean Extract. The DETBA values with the combination of soybean extract and linoleic, linolenic, or arachidonic acid are shown in Figure 4. External addition of such unsaturated fatty acids to the soybean extract induced a pronounced increase in DETBA value in the $L_{123(S)}$ and $L_{123(F)}$ soybean extracts and did so to some extent in the L_1, L_2, and L_3 soybean extracts. However, in the case of L_0, the increase was not observed.

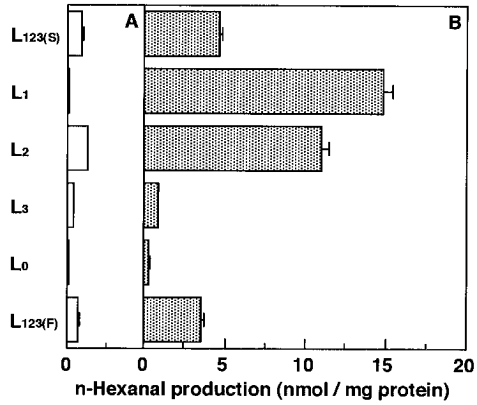

Figure 5. Effects of the external addition of linoleic acid on the production of *n*-hexanal in soybean extract. The reaction mixture in a total volume of 1 mL contained 100 mM phosphate buffer (pH 7.0), 1 mM sodium linoleate, and soybean extract. Incubation was done at 25 °C for 10 min, and the obtained sample was subjected to *n*-hexanal assay. Results are expressed as means of three experiments: (A) absence of linoleic acid; (B) presence of linoleic acid.

***n*-Hexanal Production with the Combination of Linoleic Acid and Soybean Extract.** The effects of external addition of linoleic acid on the production of *n*-hexanal in soybean extract under the reaction condition at pH 7 are shown in Figure 5. In the absence of linoleic acid, *n*-hexanal production was the highest in the L_2 soybean extract, followed by the $L_{123(S)}$ and $L_{123(F)}$ soybean extracts, and was the lowest in the L_0 soybean extract (Figure 5A). This result agreed with the results obtained with homogenates prepared from soybean flour (Nishiba et al., 1995).

External addition of linoleic acid enhanced the *n*-hexanal production with soybean extracts containing the L-1 and/or L-2 isozyme. The enhancing effect with the L_2 soybean extract can be explained from the evidence that L-2 is responsible for *n*-hexanal production (Matoba et al., 1985a). Although the highest *n*-hexanal production in the presence of linoleic acid was obtained in the L_1 soybean extract, this is probably due to the reaction pH. If the pH in the reaction mixture containing the L_1 soybean extract and linoleic acid was varied to pH 6 instead of pH 7, *n*-hexanal production was 2.54 nmol/mg of protein. The *n*-hexanal production in the soybean extract from $L_{123(S)}$ and $L_{123(F)}$ was lower than that from L_2. Also, the *n*-hexanal production in the L_3 soybean extract was lower than that in the L_1 soybean extract. This result is probably due to the effect of L-3 in reducing *n*-hexanal production (Hildebrand et al., 1990; Moreira et al., 1993).

DISCUSSION

In making food products with the combination of soybean seeds (rich in lipoxygenases) and other food ingredients (rich in unsaturated fatty acids), the amount of hydroperoxide produced in the food products should be lowered because the oxidation of unsaturated fatty acids by lipoxygenases in foods leads to the development of off-flavor and bitter taste as well as nutritional damage. The level of *n*-hexanal is also important because *n*-hexanal has a low olfactory threshold and creates great difficulties in the production of acceptable food products. Thus, we used two parameters in this study, DETBA value (as an index of hydroperoxide production) and *n*-hexanal production, for selection of the most suitable soybean seeds for combination with other food ingredients.

Among various lipoxygenase-lacking soybeans, L_0 soybeans have the lowest levels of both DETBA values and *n*-hexanal production. Furthermore, the low DETBA value was maintained over a wide range of pH and temperature and was not influenced by the species of unsaturated fatty acids added. Therefore, L_0 soybeans can be expected to be an excellent soybean source for making soybean products with the combination of soybeans and other food ingredients.

Such superior characteristics could not be completely obtained from normal and other soybeans lacking lipoxygenase. To suppress the linoleate oxidation, it is necessary to provide a treatment based on the nature of the lipoxygenase isozymes contained in the soybeans. Low DETBA values could be obtained if the pH conditions during linoleate oxidation by soybean extract were at a pH below 6.0 in L_1 and L_3 and at a pH above 8.0 in L_2. Low DETBA values could also be obtained if the temperature conditions were low (4 °C), or high (70 °C) for L_1 and L_3, high (50 °C) for L_2, and high (70 °C) for $L_{123(S)}$ and $L_{123(F)}$. However, under these pH and temperature conditions, except for their region, the increase in the DETBA value could not be suppressed completely. The existence of such regions will become a limiting factor in the utilization of normal soybeans and those lacking lipoxygenase for making food with the combination of soybeans and other food ingredients. Only the region in which there is no increase in the DETBA value can be adapted to food processing. The increase in DETBA value with normal soybeans and those lacking lipoxygenase also occurred when using not only linoleic acid but also linolenic acid or arachidonic acid as the substrate. Furthermore, when the soybean extract and linoleic acid were mixed, *n*-hexanal was produced in normal soybeans and those lacking lipoxygenase except for L_3.

As mentioned, soybeans partially lacking lipoxygenase have some weak point for making foods with the combination of soybeans and other food ingredients. In contrast, the soybeans completely lacking lipoxygenase possess traits that can eradicate such problems. The fact that L_0 soybeans and unsaturated fatty acids could be mixed will expand the use of soybean seeds as food ingredients. We expect that new types of soybean products with the combination of soybeans and other food ingredients containing unsaturated fatty acid can be created with the L_0 soybeans.

ACKNOWLEDGMENT

We are grateful to Dr. K. Kitamura (National Agriculture Research Center, Japan) for the gift of soybean cultivars Yumeyutaka and Kanto 102.

LITERATURE CITED

Ames, B. N. Dietary carcinogens and anticarcinogens. *Science* **1983**, *221*, 1256−1264.

Axelrod, B.; Cheesbrough T. M.; Laakso S. Lipoxygenase from soybeans. *Methods Enzymol.* **1981**, *71*, 441−451.

Davies, C. S.; Nielsen, N. C. Genetic analysis of a null-allele for lipoxygenase-2 in soybean. *Crop Sci.* **1986**, *26*, 460−463.

Furuta, S.; Nishiba, Y.; Suda, I. A new method for judging deterioration of stored garden peas, an index of lipid peroxidation. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 111−112.

Hajika, M.; Igita, K.; Kitamura, K. A line lacking all the seed lipoxygenase isozymes in soybean [*Glycine max* (L.) Merrill)] induced by gamma-ray irradiation. *Jpn. J. Breed.* **1991**, *41*, 507−509.

Hajika, M.; Kitamura, K.; Igita, K.; Nakazawa, Y. Genetic relationships among the genes for lipoxygenase-1, -2 and -3 isozymes in soybean [*Glycine max* (L.) Merrill)] seed. *Jpn. J. Breed.* **1992**, *42*, 787−792.

Haumann, B. F. Health implications of lipid peroxidation. *Int. News Fats Oils Relat. Mater.* **1993**, *4*, 800−810.

Hildebrand, D. F. Lipoxygenases. *Physiol. Plant.* **1989**, *76*, 249-253.

Hildebrand, D. F.; Hymowitz, T. Two soybean genotypes lacking lipoxygenase-1. *J. Am. Oil Chem. Soc.* **1981**, *58*, 583−586.

Hildebrand, D. F.; Hamilton-Kemp, T. R.; Loughrin, J. H.; Ali, K.; Andersen, R. A. Lipoxygenase 3 reduces hexanal production from soybean seed homogenates. *J. Agric. Food Chem.* **1990**, *38*, 1934−1936.

Kaneda, T.; Sakai, H.; Ishii, S. Nutritive value or toxicity of highly unsaturated fatty acids II. *J. Biochem.* **1995**, *42*, 561−573.

Kitamura, K.; Davies, C. S.; Kaizuma N.; Nielsen, N. C. Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. *Crop Sci.* **1983**, *23*, 924−927.

Kitamura, K.; Kumagai, T.; Kikuchi, A. Inheritance of lipoxygenase-2 and genetic relationships among genes for lipoxygenase-1, -2 and -3 isozymes in soybean seeds. *Jpn. J. Breed.* **1985**, *35*, 413−420.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Matoba, T.; Hidaka, H.; Narita. H.; Kitamura, K.; Kaizuma, N.; Kito, M. Lipoxgenase-2 isozyme is responsible for generation of *n*-hexanal in soybean homogenate. *J. Agric. Food Chem.* **1985a**, *33*, 852−855.

Matoba, T.; Hidaka, H.; Kitamura, K.; Kaizuma, N.; Kito, M. Contribution of hydroperoxide lyase activity to *n*-hexanal formation in soybean. *J. Agric. Food Chem.* **1985b**, *33*, 856−858.

Moreira, M. A.; Tavares, S. R.; Ramos, V.; Barros, E. G. Hexanal production and TBA number are reduced in soybean [*Glycine max* (L.) Merr.] seeds lacking lipoxygenase isozymes 2 and 3. *J. Agric. Food Chem.* **1993**, *41*, 103−106.

Nishiba, Y.; Furuta, S.; Hajika, M.; Igita, K.; Suda, I. Hexanal accumulation and DETBA value in homogenate of soybean seeds lacking two or three lipoxygenase isozymes. *J. Agric. Food Chem.* **1995**, *43*, 738−741.

Rackis, J. J.; Sessa, D. J.; Honig, D. H. Flavor problems of vegetable food proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 262−271.

Suda, I.; Furuta, S.; Nishiba, Y. Fluorometric determination of a 1,3-diethyl-2-thiobarbituric acid-malondialdehyde adduct as an index of lipid peroxidation in plant materials. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 14−17.

Suda, I.; Hajika, M.; Nishiba, Y.; Furuta, S.; Igita, K. Simple and rapid method for the selective detection of individual lipoxygenase isozymes in soybean seeds. *J. Agric. Food Chem.* **1995**, *43*, 742−747.

Received for review June 28, 1995. Accepted September 28, 1995.[⊗] This study was supported by Grant-in-Aid of Preceded Research Program provided by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan (PRP94-6-2).

JF950395J

[⊗] Abstract published in *Advance ACS Abstracts*, December 1, 1995.